PHOTOREACTIVATION AND ULTRAVIOLET INACTIVATION OF MYCOBACTERIA IN AIR

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PHOTOREACTIVATION AND ULTRAVIOLET INACTIVATION OF MYCOBACTERIA IN AIR

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SUMMARY

Apparatus was designed and assembled to suspend bacterial cells into an aerosol from a liquid suspension, irradiate the aerosolized cells in a uniform manner by known quantities of 253.7 nm ultraviolet radiation, and collect the irradiated cells on a nutrient growth medium for photoreactivation treatments, incubation and enumeration.

This study revealed that certain mycobacteria may be photoreactivated after exposure to far UV. Bacteria may show species differences in sensitivity to killing by UV while in an airborne state. It was also found that relative humidity affects the sensitivity of bacteria to UV, and in general, the lower the humidity, the more sensitive.

CHAPTER I

INTRODUCTION

The study of the response of bacterial cells to ultraviolet (UV) radiation began as long ago as 1877 when bacteria were found to be inactivated by sunlight (Downes and Blunt, 1877). In the years following this discovery, the amount of information on the effects of UV grew rapidly, but the understanding of the mechanisms producing these effects did not begin to appear until 1928. Gates (1930) reported that the wavelengths most effective in killing Escherichia coli and Staphylococcus aureus were similar to wavelengths absorbed by nucleic acids. Evidence for the chemical basis of UV effects on nucleic acids had begun to be described in the late 1940's, and the biological importance of DNA altered by UV soon became apparent (Smith and Hanawalt, 1969).

UV radiation in the "far UV" range (200 nm to 300 nm) is maximally absorbed by nucleic acids at 260 nm and produces its effects by excitation of bonding electrons to break or to cause chemical changes within a target molecule. The primary biological target of far UV is considered to be DNA for several reasons. Nucleic acids and proteins are molecules of great biological importance which absorb strongly in the

far UV range (Jagger, 1969). A large proportion of the cell material is protein and the loss of a few protein molecules would seldom produce any serious effects. On the other hand, damage at a single site on a DNA molecule could potentially lead to permanent change or death. The action spectrum of inactivation of many species of bacteria resembles the absorption spectrum of nucleic acids (Jagger, 1969; Webb, 1965; Zelle, 1955). In the 240 to 280 nm wavelength range, nucleic acids absorb much more strongly than proteins and are the most important molecular absorbers in bacterial cells (Jagger, 1969) even though nucleic acids occur in smaller quantities than proteins. There is no direct evidence that damage to RNA is not an important contributor to UV killing effects in bacteria, but since the DNA contains the genetic information, it is strongly suspected to be the more significant target (Jagger, 1969).

Pyrimidine dimers formed by far UV are the most important lesions known to occur in DNA. The bases are the primary sites of UV absorption in nucleic acids, and the pyrimidine bases absorb about 10 times more strongly than the purines (Jagger, 1969). Thymine dimers and various other pyrimidine dimers have been isolated from UV-irradiated DNA and from whole irradiated bacterial cells. Thymine dimers in DNA have been found to block DNA synthesis during in vitro experiments and in whole cells (Haynes, 1966; J. K. Setlow, 1966a). Other types of UV damage to DNA include chain breaks,

cross-links to DNA, cross-links to proteins, and hydration products of pyrimidines. Except for the hydration products these kinds of lesions are considered not to be biologically important in inactivation. Very high doses are required to produce chain breaks and cross-links when compared to doses needed to kill most bacteria (J. K. Setlow, 1966a; Smith and Hanawalt, 1969). These types of damage probably contribute to kill by UV in highly UV-resistant strains such as Micrococcus radiodurans. Based on the experimental evidence listed above, pyrimidine dimers are the only known UV-induced lesions which have been implicated in the observed biological effects of far UV irradiation of bacteria, but there is no direct proof that they cause these effects (Haynes, 1966; Jagger, 1969). Thymine dimers are produced in larger numbers than other types of dimers and are often credited with being the most important type of lesion leading to bacterial mutation and death following far UV irradiation.

The ability of a cell to survive radiation damage depends on its ability to cope with the damage, either by bypassing the lesion or by repairing it. Photoreactivation (PR) after UV irradiation is one repair phenomenon known to occur in many cells and was discovered in 1948 by Kelner during a study of Streptomyces griseus for UV-induced mutation (Rupert, 1964). It has since been demonstrated that many types of cells show PR and other recovery phenomena such as photoprotection, liquid holding recovery, and heat

reactivation (Hanawalt, 1968; Zelle, 1955). Although there is much information available concerning UV inactivation and reactivation of bacteria and other cells, there are few reports on the response of bacteria to quantitated UV exposure while in an airborne state, or on PR of such bacteria that have been exposed to killing doses of UV.

Bacterial cells in a dried or partially dried state or in an unfavorable environment might be expected to respond quite differently to stresses than cells in a wet environment. Drying a cell changes the configuration of large biological molecules by removing water molecules bound as part of their structures. Functional groups are also dehydrated and can then interact when the whole molecule changes orientation upon drying (Webb, 1965). These configurational and chemical alterations may result in drastic changes in the functions of many molecules of great importance to the cell. Because of resultant dehydration, an airborne cell may respond differently to various physical or chemical insults, and it should be worthwhile to compare responses of airborne bacterial cells with those similarly treated in liquid states.

Much of UV damage appears to be caused by removal or reorientation of bound water in important molecules and the formation of water addition products. Energy migrations are needed to displace water by UV. This becomes more difficult if more water is present, especially if displaced water molecules are quickly replaced from the environment (Webb,

1965). Published information regarding responses of airborne mycobacteria to killing by UV radiation and the occurrence of recovery processes in such irradiated cells is indeed scarce. Several reports regarding other genera are available (Kethley and Branch, 1972; Riley, et al., 1972; Webb, 1961) but these bacteria have been shown to be much more sensitive to UV than the mycobacteria when irradiated in the liquid state (Collins, 1971; David, et al., 1971; Sellers et al., 1970). Since germicidal lamps which emit primarily 253.7 nm UV are used for air disinfection, more information concerning responses of airborne cells to UV kill and conditions favorable to recovery is needed. The purpose of this research was to provide some information about UV killing and photo-recovery of mycobacteria exposed to 253.7 nm radiation while in an airborne state.

Photoreactivation might best be defined as the reduction in the response of a biological system to UV irradiation by post-treatment with radiation of longer wavelengths than the response-inducing radiation. There are several types of PR described in the literature, each of which have different action spectra peaks and different temperature and dose rate dependencies. Direct PR and indirect PR are the best known types and have been found to occur in many single-celled organisms.

Direct PR has been the most extensively studied, both on a subcellular level and with whole cells. The mechanism

of direct PR is considered to be enzymatic since this type of PR in whole cells and in extracts from photoreactivable cells shows temperature dependence and dose rate dependence to the photoreactivating light. Extracts from E. coli and yeasts have been obtained which can photoreactivate UV damaged DNA in vitro (Setlow and Boling, 1963). These extracts show heat lability and are susceptible to proteolytic enzymes. The mechanism of this type or PR is thought to follow the Michaelis-Menten reaction scheme for repair of DNA (Hanawalt, 1968; J. K. Setlow, 1966b) whereby the photoreactivating enzyme initially binds specifically with UV damaged DNA at pyrimidine dimer sites. Illumination of these enzyme-DNA sites splits the dimers, frees the enzyme and produces repaired DNA and free active enzyme. When direct PR is said to show a "dose rate dependence", the amount of recovery seen in UV irradiated cells or DNA which have been photoreactivated with the same dose increases with lower dose rates or intensities. The recovery efficiency is better at low dose rates. When temperature dependence occurs, more efficient recovery per unit dose occurs at higher temperatures within the biokinetic range of enzymatic activity than at lower temperatures during PR (Jagger, 1964; Jagger and Stafford, 1965).

Indirect PR is a recovery phenomenon which may be seen in some organisms not possessing a direct PR system. It is considered to be nonenzymatic, does not act directly

on the DNA, shows little or no dose rate or temperature dependence and has an action spectrum with a maximum at 334 nm compared to a maximum of 405 nm for direct PR. Indirect PR exhibits an action spectrum similar to that of photo-induced growth delay and, therefore, the mechanism may be a delay in growth and division, which results in allowing more time available for repair mechanisms to operate (Jagger, 1964; Jagger, 1969; Jagger, et al., 1969; Smith and Hanawalt, 1969).

Evidence that PR acts primarily on thymine dimers includes both *in vitro* studies with extracts from photoreactivable cells and UV irradiated DNA in which the amount of thymine dimers is decreased in the presence of photoreactivating enzyme and light (J. K. Setlow, 1966a; J. K. Setlow, 1966b; Wulff and Carrier, 1962) and in *in vivo* experiments in which irradiated strains of *E. coli* were found to contain fewer thymine dimers after PR (Riklis, 1965; Setlow and Carrier, 1964; Smith and Hanawalt, 1969). All the types of pyrimidine dimers can be monomerized by photoreactivating enzyme, but it seems that the thymine dimer is most efficiently monomerized by this system (Jagger, 1969; J. K. Setlow, 1966a; J. K. Setlow, 1966b). Other restoration phenomena include photoprotection, liquid holding recovery, and less well known recovery phenomena. Photoprotection is the reduction in response to far UV by pretreatment of irradiated cells with longer wavelengths

than the response inducing radiation. Photoprotection seems to be the same phenomenon as indirect PR (Jagger, 1969; Setlow and Boling, 1963) except that treatment is prior to exposure to the killing UV rather than afterward. Photoprotection and indirect PR exhibit the same temperature, wavelength and dose rate dependencies (Jagger, 1964). Liquid holding recovery is the reduction in response to UV exposure effected by "holding" the cells in a non-nutrient medium for a period of time after the UV irradiation. It is thought to delay growth and thereby provide time for repair mechanisms to operate within the cell.

Killing of airborne Serratia marcescens was reported by Riley and Kaufman (1972) for 253.7 nm radiation and by Webb (1961; 1965) for wavelengths longer than 280 nm. Webb found that airborne S. marcescens inactivated by these longer wavelengths could be photoreactivated. PR was also found to occur in liquid suspensions of Mycobacterium smegmatis (Sellers, et al., 1970), M. tuberculosis, M. marinum and M. kansasii (David, et al., 1971), when exposed to 253.7 nm radiation.

The problem of this investigation was to determine if different species of bacteria exhibit dissimilar sensitivities to killing by 253.7 nm UV radiation if exposed while in an airborne state, and if these organism can be photoreactivated after such exposure as is known to occur when such organisms are similarly treated while in a liquid suspension.

CHAPTER II

MATERIALS AND METHODS

Equipment

The apparatus used in this study was designed and assembled to suspend bacterial cells into an aerosol from a liquid suspension, irradiate the aerosolized cells in a uniform manner by known quantities of 253.7 nm ultraviolet radiation, and collect the irradiated cells on a nutrient growth medium for treatment, incubation and enumeration.

Figure 1 shows the quantitative exposure system with each related component numbered for reference to the following descriptions. The components are described in the order that they are encountered by the aerosolized particles containing the bacterial cells as they pass through the system. Items 12 and 13 are not shown.

(1) Bird 500cc Inline Long Term Nebulizer (Bird Corporation, Palm Springs, California 92262). Figure 2 shows the air and particle flow through the nebulizer. For these experiments, the capillary tubing was replaced with 1/4 inch O.D. by 1/8 inch I.D. Tygon tubing with a 26 gauge 3/4 inch B-D hypodermic needle fitted into the uptake end of the tube to control the liquid flow to the nebulizer jet. The I.V. ports were filled with silicone rubber sealer to

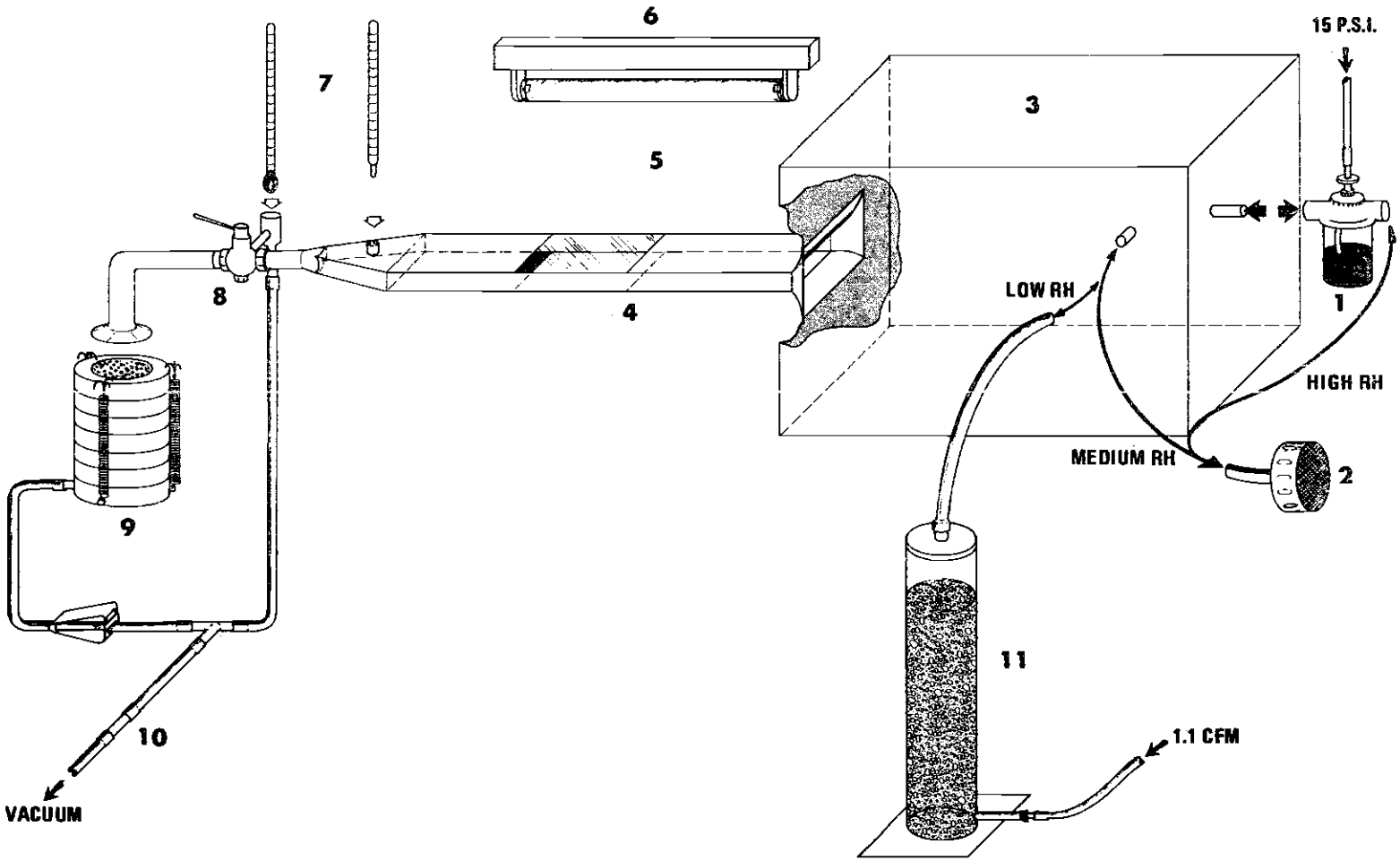

Figure 1. Quantitative Exposure System.

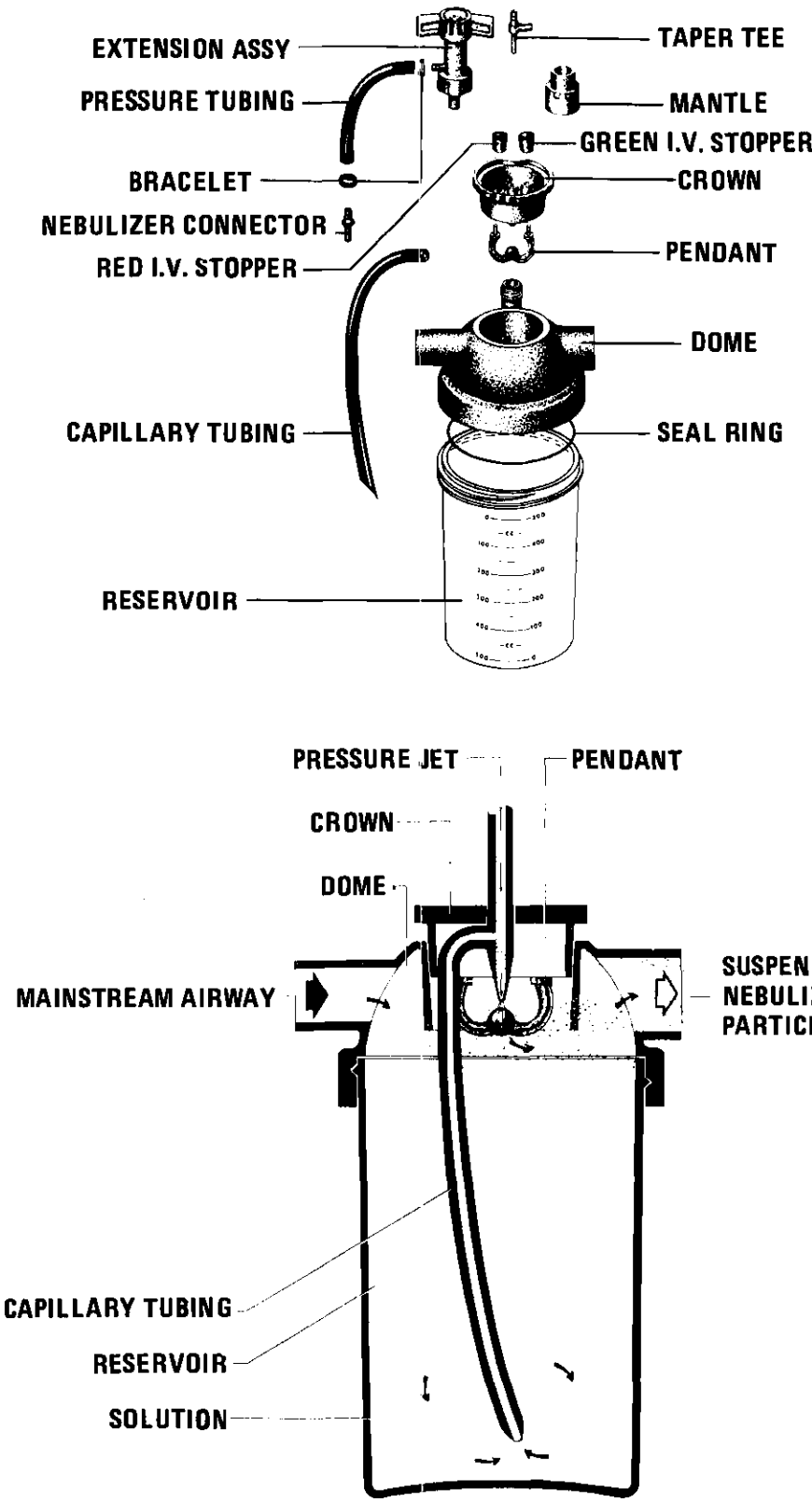

Figure 2. Bird 500cc Long Term Inline Nebulizer.
(Figure courtesy of Bird Corporation)

eliminate pockets in which contaminant organisms might grow between uses. After each run the nebulizer was disassembled, soaked in 100 ppm NaOCl disinfectant (made from commercial bleach), washed with hot water containing Alconox, rinsed thoroughly, and stored at 42°C to dry before the next use. It was not necessary to decontaminate the Bird with stronger treatments if these steps were followed. The nebulizer was operated at 1.05 newtons/m^2 (15 psig) air pressure which effected a flow of 4.6 x 10^3 cm^3/min (0.16 ft^3/min) through the jet. Under these conditions, with the #26 needle in the uptake tube, the nebulizer consumed 10 ml of bacterial suspension per hour when operated in the low and medium relative humidity (RH) ranges and about 15 ml per hour when operated in the high RH mode (due to greater volume of air passing through the nebulizer when operating at high RH).

(2) Super Micro-Toxisol R-57 Filter (American Optical Company, Safety Products Division). Room air drawn into the system was cleared of airborne biological contaminants by attaching one filter to the intake port of the stilling chamber for the medium RH mode or to the intake port of the nebulizer for the high RH mode.

(3) Stilling chamber. A stilling chamber, having dimensions of 45, 43 and 34 cm was constructed from 1/4 inch methyl methacrylate plastic, the seams being cemented with ethylene dichloride solvent and each seam was also sealed on the inside of the chamber with Dow Corning Silicone

Rubber Sealer. A hole was cut in the end opposite the laminar flow exposure section to provide access to the chamber for cleaning. This hole was covered with a piece of 1/4 inch methyl methacrylate plastic and sealed with Sears Silicone Rubber Cement. Although this cement provides an airtight seal, it does not adhere to the plastic as firmly as the Dow Corning sealer, which allows the cover to be removed easily. All inside surfaces of the stilling chamber were painted with flat black spray can paint, smoothed by rubbing with wet 320 grit emery paper and finally wet 600 grit emery paper.

(4) Laminar flow section. This section was constructed of methyl methacrylate plastic and had internal dimensions of 2.1 cm high by 15.2 cm wide. This provided a velocity of 15.2 cm/sec when the flow rate through the slot was 2.89×10^4 cm^3/min (1 ft^3/min). The length of the entire laminar flow section was 46 cm with a 15.2 cm long exposure section in the center. To obtain laminar flow in this section, each wall of the slot at the input end curved outward into a 5.6 cm radius bend with the corners meeting to form a rectangular funnelled "flange". This flange was mounted parallel to and flush with the inner surface of one wall of the stilling chamber which had a rectangular hole cut to accept the flange.

The internal surfaces were treated as in the stilling chamber. The dimensions for the system that would provide

laminar flow were provided by Professor James E. Hubbartt (Aerospace Engineering, Georgia Institute of Technology, Atlanta, Georgia 30332). The system was checked for turbulence by pulling smoke from a ventilation smoke tube through an open intake port while operating the system at a 2.89 x 10^4 cm^3/min flow. Visual examination of the smoke puffs passing between the UV windows revealed no perceptible turbulence and the smoke fronts passed between the windows without change in shape.

(5) Ultraviolet windows; Dow Corning filters, CS No. (color specification number) 9-54 (Corning Glass Works, Corning, New York). These windows were made of Dow Corning 7910 glass (98% fused quartz), had dimensions of 16.5 cm square by 2 mm thick, and had a specified transmission of more than 70% at 254 nm (Corning Glass Works, 1965). The windows were mounted in the center of the laminar flow section parallel and opposite to each other and flush with the inner surfaces of the 15.2 cm walls of the laminar flow section. These windows were sealed externally with Sears Silicone Adhesive Rubber Cement (Sears Roebuck and Co., Chicago, Illinois 60607) and readily could be removed for cleaning by cutting away the sealer with a razor blade. The air velocity through the laminar flow section, and therefore between the windows, was 15.2 cm/sec, resulting in a one second exposure from the germicidal lamps to any aerosol passing through. All pieces making up the laminar

flow section were closely fitted to make the surfaces smooth at the edges of the UV windows and at the flange surface attaching the input end of the slot to the stilling chamber.

(6) GE G15T8 germicidal lamps (General Electric Co., Large Lamp Department, Nela Park, Cleveland, Ohio 44112). The specified output of these lamps is 45% 253.7 nm energy and 2% visible radiation with the remaining energy dissipated into heat. The measured outputs of the lamps used for this study were within 10% of specifications (General Electric Co., 1967). The lamp fixtures were mounted on a stand above the UV windows and could be adjusted to a height necessary to give the desired intensity.

(7) Thermometers. Wet and dry bulb glass mercury thermometers were used to measure the RH inside the system at the laminar flow section. The dry bulb was inserted through a port at the end of this section, and the wet bulb was mounted below the control valve where the air flow was at a sufficient velocity to evaporate the wet bulb moisture and where the presence of a wetted wick would not interfere with the experimental aerosol.

(8) One-half inch three-way brass valve. A valve was used to switch the air stream between the sampler and by-pass vacuum line, maintaining a continuous flow through the system at all times during a run.

(9) Andersen sampler (Anderson Samplers & Consulting Service, Providence, Utah). Six stage Andersen samplers

were used with 27.0 ml of nutrient agar in the plates. The samplers were used at a flow rate of 4.8×10^3 cm^3/sec (1 ft^3/min). The construction and use of this sampler is described in detail by Andersen (1958).

(10) Critical pressure ratio orifice. A vacuum pump was used at this end of the system operating at a pressure of 0.33 newtons/m^2 (20 in Hg vacuum) with a critical pressure ratio orifice between the pump and the system, providing a constant flow of 4.8×10^3 cm^3/sec through the system.

(11) Drying column for low RH conditions. The body of this column was constructed of a length of rigid plastic tubing which was 61 cm long with a diameter of 12.5 cm. For the drying agent, seven to eight pounds of TEL-TALE Silica Gel Disiccant, mesh size 6-16, an indicator silica gel, were used in the column. The column provided approximately 10% RH air at a rate of 3.2×10^4 cm^3/min. The air was forced from the bottom of the column to the outlet under pressure and was monitored with a flow meter (not shown).

(12) Blak-Ray UV meter (Ultra-Violet Products, Inc., San Gabriel, California 91778). Ultraviolet intensity measurements were made with this hand-held meter using the appropriate sensor. The J-225 meter is calibrated for short wave germicidal lamps. The J-221 meter is calibrated for long wave UV and was used to measure the black light lamps

used for PR treatments. The meter calibration was by the manufacturer and was confirmed by Karl Branch (Center for Disease Control, Atlanta, Georgia 30333) by sodium ferroxalate actinometry following the procedure outlined by Parker (1968).

(13) Photoreactivating illumination sources. Three types of sources were used for photoreactivating light, each with a different wavelength range: (a) Three Sylvania F40-BL fluorescent lamps, mounted in a fixture 15 cm above the Andersen plates during treatments, gave intensities of 600 $\mu W/cm^2$ and 1800 $\mu W/cm^2$. The 600 $\mu W/cm^2$ intensity was obtained by placing five layers of cheesecloth between the lamps and sample plates. The output of these lamps is primarily near UV between 300 nm and 400 nm (Jagger, 1969). (b) Three well used Ken-Rad 40 watt rapid start fluorescent lamps mounted in a fixture 35 cm above the treated plates served as a source similar to the one used by Sellers, et al. (1970) and provided an intensity of approximately 150 foot-candles to the plates. The spectral output of cool white fluorescent lamps is from 400 nm to 700 nm (Jagger, 1969). (c) A third PR source was a 100 watt 130 volt inside frost Sylvania incandescent bulb mounted in a 30 cm diameter parabolic flood reflector. The reflector was lined with aluminum foil, shiny side outward, to increase the intensity to about 180 foot-candles when placed 30 cm above the plates. This source was intended to duplicate that used by David, et al. (1971).

Operation of Equipment

Control of Temperature and Relative Humidity

Overall temperature control was afforded by the air-tempering equipment of the Aerobiology Laboratory workroom in which all experiments were conducted. In this workroom ambient air was maintained at a temperature of 21-22°C and at 35-45% RH. During any one experimental run the room RH and the system RH did not vary more than three or four per cent.

Airflow into the stilling chamber was conducted through one of three paths, depending on the humidity range desired for a particular experiment. For high RH the stilling chamber port was stoppered and room air was drawn through a filter into the airway of the nebulizer (see Figure 1) and then into the stilling chamber. This resulted in 80 to 88% RH in the system. For low and medium RH the input of the nebulizer airway was stoppered and air was drawn directly into the stilling chamber through the port at the side of the chamber near the Bird nebulizer (Figure 1). For medium RH conditions, room air was drawn via this port through a filter into the chamber, resulting in 50 to 60% RH. For the low RH range, room air was drawn from the top of the drying column (part 11 in Figure 1) into the stilling chamber. To prevent possible collapse of the system, air was forced by pressure from the bottom through the column at 5.2×10^3 cm^3/sec, a flow which was in excess of the

amount required by the rest of the system. The surplus dry air was allowed to exhaust through 10 holes three millimeters in diameter drilled into the output tube of the drying column, which eliminated forcing excess air into the stilling chamber. The air drawn into the stilling chamber from the drying column was at 8 to 10% RH and when mixed with the spray from the nebulizer, produced in the exposure end of the system of 28 to 32% RH.

The total air flow through the system was maintained at 4.8×10^3 cm^3/sec (1 ft^3/min), with 7.7×10^2 cm^3/sec contributed by the nebulizer spray and the remainder coming from outside the stilling chamber, from the room via a filter or from the drying column.

Humidity Measurements

At the downstream end of the laminar flow section a removable thermometer was stoppered into a port for dry bulb temperature readings. A removable wet bulb thermometer was placed below the control valve where the air flow was at a sufficient velocity to evaporate water from the wet bulb sock. The wet bulb readings were confirmed to be minimal with this configuration by passing plain room air through the system at 4.8×10^3 cm^3/sec and comparing the wet bulb temperature to a forced air psychrometer in the room. The wet bulb sock was cleaned frequently to remove materials accumulated from the aerosol. Both thermometers were removed during sampling.

Irradiations

The killing radiation to which the aerosol was exposed in the laminar flow section was obtained by one of three arrangements: (1) one lamp adjusted to a height of 26 cm to give 300 $\mu W/cm^2$, (2) one lamp at a height of 17 cm to give 500 $\mu W/cm^2$, or (3) from two lamps at 11 cm to give 1500 $\mu W/cm^2$ intensity. The lamps were positioned over the windows and the height was measured from the top surface of the upper window to the lamp tube. The UV intensity variation across the window was 20% or less. Since the time spent between the UV windows by any particle passing through was one second, the dose could be calculated by multiplying the intensity by one second.

Intensity measurements were made with the J-225 UV meter after a five minute lamp warmup. The sensor was placed directly on the windows for the measurements. Two measurements were taken over the top window and two were taken beneath the lower window. One of these two readings was made with a piece of 0.8 mm thick Pyrex glass used as a filter to filter out any UV and provide a measure of visible or infrared light being read by the meter. The second measurement was made directly with the sensor unfiltered and corrected by subtracting the filtered measurement. The correction was about 10% of the unfiltered reading. The average of the corrected upper and lower UV intensities was assumed to be the actual 253.7 nm intensity or the radiation

to the aerosol inside the system between the windows. About 25% of the UV was absorbed by each window which agrees with the transmission properties specified for these filters (Corning Glass Works, 1965). The lamps and exposure section of the system were shrouded with six mil black polyethylene plastic to protect personnel from the UV and to prevent room light from entering the system at the UV windows.

Sampling

The three-way control valve could be switched to direct air through a bypass hose or through the sampler. The sampler vacuum hose was pinched off by a hose clamp simultaneously with switching the valve to the sampling position. The air flow was never interrupted, and the chamber conditions were maintained in a steady state at all times during a run.

While samples were being taken, the entire flow of aerosol through the system passed through the Andersen samper which was operated at 4.8×10^3 cm^3/sec. The plates were prepared one to five days before use and were filled with 27.0 ml of nutrient agar (Difco) each. Because the system was operated continuously at 1.0 ft^3/min, samples of 1.0, 2.0, 4.0, or 8.0 ft^3 were obtained by taking air through the Andersen for periods of 1.0, 2.0, 4.0 or 8.0 minutes

Cultures

The test organisms used for the study were M. smegmatis,

ATCC 607, and M. phlei, Gordon's No. 644-5. Both were obtained from Dr. Hugo David (Center for Disease Control, Atlanta, Georgia 30333) and were the same as those used by him for his work (David, et al., 1971). Stock cultures were maintained on 7H10 agar (Difco Laboratories, Detroit 1, Michigan) slants in 20 mm by 150 mm screw cap culture tubes and were transferred every four months by streaking into fresh slants. The stock slants were grown at 36°C for four days, then stored at 5°C.

Working cultures were maintained by serial transfer of 0.5 ml of inoculum into approximately 40 ml of 7H9 broth in a 125 Erlenmeyer flask every 42 to 52 hours. The 7H9 broth was prepared in 200 ml quantities in the following manner: One gram 7H9 broth base (Difco) and 0.1 g Tween 80 (Difco) and 180 ml of distilled water were mixed in a 500 ml Erlenmeyer flask stoppered with a cotton plug. This broth base was steam sterilized in an autoclave at 15 psig and 120°C for 15 mintues, and stored at room temperature in the same flask until needed. When fresh medium was needed, ADC additive (Baltimore Biological Laboratories, Cockeysville, Maryland 21030) was aseptically added to one flask of broth base and the mixture dispensed into five sterile, dried, cotton plugged 125 ml Erlenmeyer flasks in approximately 40 ml amounts. The small flasks of medium were incubated for 24 to 48 hours at 36°C to detect contamination which might have been acquired in dispensing. The culture medium was

then stored until used, but for no more than one week.

After inoculation, the medium was incubated at 36°C with horizontal shaking for 42 to 48 hours to place the culture well into stationary phase. The shaking rate was at 200 excursions/min in 2.5 cm circles. The flasks were covered with aluminum foil during the entire incubation period to exclude light.

Preparation of the Aerosolization Suspension

To obtain single cells for aerosolization, the raw culture was serially filtered by vacuum through 8µm, 5µm, and finally 3µm pore size 47 mm diameter filters (Millipore Corporation, Bedford, Massachusetts 01739). The filtering glassware was sterilized before use. The smaller M. phlei cells readily passed through all three filters. The raw M. smegmatis clogged the 8µm filter after 10 to 15 ml had passed through, but the filtrate passed readily through the succeeding filters of smaller pore size. For both species, microscopic examination of the final filtrate by phase contrast confirmed that only single cells were present.

One ml of the final filtrate was added to 100 ml of 0.2% bovine albumin in a milk dilution bottle. The suspending medium was prepared shortly before use by the aseptic addition of 0.6 ml sterile 35% Fraction V bovine albumin (Nutritional Biochemical Corporation, Cleveland, Ohio 44128) to 100 ml sterile distilled water in a milk dilution bottle.

For the aerosolization suspension, one ml or 10 ml of the first 1:100 dilution was added to a second bottle containing 100 ml of 0.2% bovine albumin, giving $1:10^4$ or $1:10^3$ dilution of the filtrate. Spread plate counts made from the aerosolization suspension before each run showed the M. phlei, always used at a $1:10^4$ dilution, to have a concentration of approximately 2.5×10^3 cells/ml and M. smegmatis to have a concentration of approximately 450 cells/ml when diluted to $1:10^4$ and 4.5×10^3 cells/ml when diluted $1:10^3$.

Procedure

The prepared aerosolization suspension was poured into the Bird nebulizer in dimmed light, with the nebulizer and the dilution bottle containing the suspension wrapped with aluminum foil to exclude light. A small amount of the suspension was withheld for the spread plate counts. The nebulizer reservoir was immediately immersed in an ice bath within 3 cm of the nebulizer dome. Since the amount of suspension used was never more than 200 ml, the level of liquid within the nebulizer was always well below the surface of the ice bath.

A filter on the outlet tubing of the drying column was than attached to the proper port to obtain the RH desired for any one experiment. The vacuum pump was started and when the correct flow through the system was confirmed, the nebulizer jet was activated. The system was allowed to

operate for one hour for equilibration before sampling was begun. The RH measurement was recorded just before the first sample was taken. The germicidal lamps were operated for five mintues before the intensity measurement was taken and were then adjusted if necessary immediately before the first UV sample. Each sample set was taken as a group. All control, UV, and UV + PR samples for each time were completed before beginning the next set. The plates were removed from the Andersen sampler immediately after sampling was completed. In each experimental run, four sets of samples were taken, each set at one of sampling times 1, 2, 4 or 8 minutes. This series of sampling times usually gave counts of 700 to 2000 control colonies totaled from two or more sets. Each sample set consisted of one each of six samples: control (no irradiation at 253.7 nm or PR light), untreated UV, UV with 15 minutes black light at 1800 $\mu W/cm^2$, UV with 15 minutes black light at 600 $\mu W/cm^2$ PR treatment, UV with one hour under the incandescent lamp, and one UV sample treated one hour under the cool white PR light. The one hour PR plates were covered with sterile Pyrex petri covers during the treatment to prevent incidental contamination. The 15 minute black light plates were treated uncovered, but the fixture was shrouded with black polyethylene plastic film to exclude room air currents.

Each sample of six plates was wrapped with aluminum foil before incubation to prevent unwanted exposure to room

light. Control samples and untreated UV samples were wrapped in an area illuminated only by a 25 watt red light bulb. All plates were incubated at 36°C for four days before counting.

Treatment of Data

After incubation, colonies were counted on each plate from the Andersen samplers and each plate count was corrected to the most probable number as described by Andersen (1958). The corrected counts of each of the six plates in a sample were totaled to obtain that sample count. For the remainder of this paper, the term "count" will refer to the corrected count and not the raw plate count. Any sample count totaling 2000 or more was not used.

For each control sample at a given sampling time, there was one sample each of untreated UV and four PR treated samples (see Procedure) of equal sampling time for comparison. The per cent survivals were calculated by summing all control sample counts and expressing the sum of all the corresponding UV or UV + PR samples as a percentage of this control total. Statistical determination of PR recovery was accomplished for each set of experimental conditions by testing the difference between the totaled UV sample counts and the total of the corresponding UV + PR samples. In each of these comparisons, the total sampling time (and, therefore, the volume of air sampled from the system) was the same for both the control sample and UV sample or UV sample and UV + PR samples. The

samples were treated as Poisson distributions, so the variance is equal to the mean. To test that two counts were different, a normal approximation was assumed (because total counts were always well over 100) and the significance ratio x/s was used, where $x = \overline{X}_1 - \overline{X}_2$, $\overline{X}$ = the count for a given sample, and $s = (\overline{X}_1 + \overline{X}_2)^{1/2}$ for the Poisson expectancy (Goldstein, 1964).

Replicability was checked by converting each sample to number viable cells/ft^3 collected and calculating the variance (Croxton, 1953) and then comparing this value to the mean of the samples being tested which is the theoretical variance if the sample distribution was Poisson.

CHAPTER III

RESULTS

Results for M. smegmatis are summarized in Table 1 which shows survival with and without photoreactivation (PR) treatments. Results of UV survival of M. phlei are summarized in Table 2. M. smegmatis showed the capability to photoreactivate after far UV irradiation at all three RH ranges examined. When the survival of untreated cells and PR treated cells of M. smegmatis which had been exposed to the same killing dose of far UV with the same temperature and RH conditions were compared, up to 32% of the inactivated cells recovered. The most effective photoreactivating source was the black light fluorescent lamps at the high intensity (1800 $\mu W/cm^2$ for 15 minutes). There was no evidence that M. phlei could be photoreactivated under any of the conditions imposed in these experiments.

In the case of M. smegmatis, the difference between the counts of untreated UV survival and the UV with the high intensity PR survival is significant at the 0.01 level except at the high RH condition at 500 $\mu W/cm^2$ UV where the kill was too small for recovery to be detectable. The designated PR survivals were significant at the 0.05 level of confidence.

All the M. phlei sample variances were within the

Table 1. Survival of Airborne M. smegmatis After Irradiation with 253.7 nm UV at Various Relative Humidities and After Exposure to Photoreactivating Light, Expressed as Per Cent of Controls

Relative Humidity	Dose		
	300 μW-sec/cm^2	500 μW-sec/cm^2	1500 μW-sec/cm^2
80-88%		Controls : 1146	Controls : 1892
		UV : 90%	UV : 40%
		UV + Hi BL: 90%	UV + Hi BL: 55%*
		UV + Lo BL: 96%	UV + Lo BL: 41%
		UV + IL : 89%	UV + IL : 52%*
		UV + CW : 97%	UV + CW : 53%*
50-60%	Controls : 464	Controls : 616	Controls : 2557
	UV : 44%	UV : 40%	UV : 6%
	UV + Hi BL: 59%*	UV + Hi BL: 53%*	UV + Hi BL: 14%*
	UV + Lo BL: 47%	UV + Lo BL: 49%*	
	UV + IL : 39%	UV + IL : 44%*	
	UV + CW : 47%	UV + CW : 42%	
28-32%	Controls : 787	Controls : 2800	
	UV : 34%	UV : 22%	
	UV + Hi BL: 55%*	UV + Hi BL: 33%*	
	UV + Lo BL: 42%*	UV + Lo BL: 30%*	
	UV + IL : 32%	UV + IL : 22%	
	UV + CW : 34%	UV + CW : 23%	

Controls = total of all unirradiated and untreated sample counts from the experimental run imposing the conditions specified in the headings.

UV = per cent survival of total of all 253.7 nm UV irradiated samples for the experimental conditions specified, without photoreactivation treatments.

UV + Hi BL = per cent survival of total of all 253.7 nm UV irradiated samples with 15 minutes black light treatment at 1800 μW/cm^2.

UV + Lo BL = per cent survival of total of all 253.7 nm UV irradiated samples with 15 minutes black treatment at 600 μW/cm^2.

UV + IL = per cent survival of total of all 253.7 nm UV irradiated samples with one hour under 100 watt incandescent bulb.

UV + CW = per cent survival of total of all 253.7 nm UV irradiated samples with one hour under cool white fluorescent bulb.

* photoreactivation statistically significant at 0.05 level.

Table 2. Survival of Airborne M. phlei After Irradiation with 253.7 nm UV at Various Relative Humidities, Expressed as Per Cent of Controls

Relative Humidity	Dose		
	300 W-sec/cm^2	500 W-sec/cm^2	1500 W-sec/cm^2
80-88%		Controls: 2498 UV : 88%	Controls: 3203 UV : 60%
50-60%	Controls: 2571 UV : 67%	Controls: 3099 UV : 45%	Controls: 7529 UV : 14%
28-32%	Controls: 2227 UV : 68%	Controls: 2001 UV : 42%	Controls: 1173 UV : 6%

Controls = total of all control counts from the experimental conditions specified in the headings.

UV = per cent survival of total of all 253.7 nm UV irradiated samples for the experimental conditions specified.

The black light, incandescent and cool white photoreactivation treated samples showed no significant recovery.

95% confidence limits expected for a Poisson distribution except in one experiment in which the UV samples at medium RH and 1500 $\mu W/cm^2$ UV intensity exhibited a high variance. Two additional experiments were conducted whereby the variance did fit the Poisson distribution and similar results were obtained. All three experiments were added together in calculating the values shown in Table 2. Two M. smegmatis sample totals, the untreated UV ("UV survival" in Table 1) at low RH and 300 $\mu W/cm^2$ UV intensity and the UV with high intensity black light PR treatment at high RH and 1500 $\mu W/cm^2$ UV intensity, had variances smaller than expected for a Poisson distribution.

Table 3 shows the dose required to kill 90% of the cells and the proportionality constant, Z, for both species at RH range. Values for S. marcescens are included for comparison.

M. phlei and M. smegmatis exhibited different sensitivities to 253.7 nm UV when irradiated as aerosols under similar conditions of temperature and RH. Of the two species, M. smegmatis proved more sensitive than M. phlei. The responses of several mycobacterial species to 253.7 nm UV irradiation while in a wet state are listed in Table 4 which shows the dose to inactivate 90% of the cells, estimated from published results (Collins, 1971; David, et al., 1971; Sellers, et al., 1907). When the results from this study are compared to these data, both species were found to be

Table 3. LD_{90} and Z Values for 253.7 nm UV Irradiated Airborne M. phlei, M. smegmatis and S. marcescens at Various Relative Humidities

Organism	28-32% RH		50-60% RH		80-88% RH	
	LD_{90}	Z	LD_{90}	Z	LD_{90}	Z
M. phlei (Gordon 644-5)	1500	0.0015	1700	0.0014	7000	0.00033
M. smegmatis (ATCC 607)	700	0.0033	1200	0.0019	4000	0.00058
S. marcescens *	50	0.046	60	0.038	--	---

LD_{90} = dose required to inhibit colony formation in 90% of irradiated organisms, given in units of $\mu W\text{-sec/cm}^2$.

Z = proportionality constant from the expression $N/N_o = e^{-ZD}$ where N/N_o = proportion of survivors and D = dose. The units of Z are $cm^2/\mu W\text{-sec}$.

* Values for S. marcescens were calculated from results reported by R. L. Riley and J. E. Kaufman, 1971, Appl. Microbiol. 23:1113-1120.

M. phlei and M. smegmatis were aerosolized from a cell suspension in 0.2% beef albumin. S. marcescens was aerosolozed from a cell suspension in 2.0% inositol.

Table 4. Dose Required to Inactivate 90% of Organisms of Several Mycobacterial Species Irradiated by 253.7 nm UV While in Wet Conditions

Organism	Reference*	Dose, μW-sec/cm^2
M. phlei	Collins	4800
M. smegmatis	David, et al.	24300
M. smegmatis	Sellers, et al.	24000
M. tuberculosis	David, et al.	5760
M. tuberculosis	Collins	2400

* Collins, F. M., 1971, Appl. Microbiol. 21:411-413.

David, H. L., Jones, W. D., Jr., and Newman, C. M., 1971, Infect. and Imm. 4:318-319.

Sellers, I. M., Nakamura, R., and Tokunaga, T., 1970, J. Gen. Virol. 7:233-247.

more sensitive to UV when irradiated in the airborne state than when irradiated in the liquid state. The exception to this may be M. phlei in an environment of high humidity.

At low RH, both were more sensitive to far W than at high RH. M. smegmatis was more sensitive than M. phlei at all three RH ranges investigated. In contrast, recent work by David (unpublished data) indicate that, when irradiated with 253.7 nm energies as a suspension in 7H9 broth, M. phlei was slightly more sensitive than M. smegmatis, under the same conditions.

CHAPTER IV

DISCUSSION

Variation is the rule in almost any biological response between and within types of organisms. It is not known for certain which factors influence sensitivity to UV kill, but hypotheses may be made which suggest areas to be investigated. Dissimilarities in response to far UV exposure among organisms are often attributed to genetic control, primarily through different efficiencies of repair of damaged DNA (Haynes, 1966; Jagger, 1969; R. B. Setlow, 1967) or through varying factors which may influence repair mechanisms that are present (Hanawalt, 1966; Smith and Hanawalt, 1969). Sensitivity to UV in bacteria has been related to the adenine-thymine content in the DNA (J. K. Setlow, 1966) and this correlation has been considered evidence, along with other observations, that dimerization of thymine is a major cause of UV kill.

Repair probably is not the only factor influencing the response to UV irradiation. Resistant strains may possess redundant genetic information or bypass systems to get around metabolic pathways interrupted by damage to the DNA. A lesion that may be lethal to one species may be unimportant to another. Out of all possible lesions produced

by an insult, sensitivity would be influenced only by the types of lesions that would disturb the metabolism of a cell.

DNA in the dried state is resistant to the formation of thymine dimers when irradiated by UV (Riklis, 1965; Riklis and Simpson, 1964; J. K. Setlow, 1966). Webb (1965) was unable to find thymine dimers in aerosolized cells killed by UV at 60% RH and only a small amount of thymine dimers in cells killed at 30% RH. Thymine dimers are often credited with being the major photoreactivable lesion formed in UV irradiated bacteria. M. smegmatis has been shown to be photoreactivable after UV irradiation in the wet state (Sellers, et al., 1970), and the results from this investigation show that M. smegmatis can also be photoreactivated after UV irradiation in partially dried (airborne) states. If thymine dimers actually do not form in significant amounts upon UV irradiation of airborne cells, then there must be some other major photoreactivable UV lesion in M. smegmatis other than thymine dimers.

The amount of photoreactivation occuring in M. smegmatis after both wet (Sellers, et al., 1970) and airborne UV irradiation is within the range of UV inactivation covered in this study (see Table 1). These comparisons imply that, in this species, the photoreactivable damage may be the same after either condition of UV irradiation, and that this damage may not be thymine dimer lesions if these dimers do not form in DNA in whole cells irradiated while in partially

dried states. However, the source (and wavelength range) that produces the most PR in the conditions of this investigation were not the same as the cool white fluorescent lamps used by Sellers, et al. (1970) and may indicate two different mechanisms of recovery, or less efficient repair with the cool white source, which is deficient in near UV. The cool white source produced little recovery in M. smegmatis which had been irradiated in the airborne state (see Table 1).

Only a few reports on UV irradiation of mycobacterial species are available, and among these, there is a large variation in the reported sensitivities (Collins, 1971; David, et al., 1971; Sellers, et al., 1970). Some of this variation may be due to condition of irradiation, i.e., David, et al. (1971) irradiated in 7H9 liquid medium, Sellers, et al. (1970) washed the cells and resuspended them in buffered solution for irradiation, and Collins (1971) irradiated the cells on agar plates. The lack of a standardized measurement technique may also contribute to these variable results.

Although comparisons between these reports cannot be too exacting, some estimates might be made. It appears that wet M. phlei is more sensitive to 253.7 nm UV than wet M. smegmatis. In the dried state, M. phlei is the more resistant. The inconsistency between the wet and dry responses of the two species may be due to different repair mechanisms and/or different lesions occuring in these species

when irradiated by far UV. The explanation for this inconsistency may, however, be more mechanical than molecular.

The higher resistance of wet M. smegmatis compared to wet M. phlei may be due to clumping of the cells in the raw culture. While determining which growth conditions would give the highest numbers of single cells for aerosolization, it was my observation that with the conditions imposed by David, et al. (1971) or Sellers, et al. (1970) in growing M. smegmatis cultures, a large proportion of the cell population would consist of small clusters of up to about 10 cells, with the rest of the cells being single. Unless special efforts were taken to break up or remove these small clumps, a significant portion of the culture was clumped and may have caused the organism to appear more resistant to killing irradiations than a true single cell suspension. M. phlei grown under the conditions described in this paper was composed of essentially single cells, with some very large clumps of several hundred or several thousand cells. The single cells far outnumbered the number of clumps, so M. phlei grown in 7H9 broth with shaking would behave as a suspension of single cells, while M. smegmatis would not. Since many bacterial species clump under various conditions of growth, it will be important to determine if this factor will affect results or comparisons of UV kill.

Another factor that might affect sensitivity is the presence of pigmentation, pigment precursors or other

absorbing material in or on the cell. An absorber within the cell would protect target molecules from UV damage only by randomly absorbing a photon before it could be absorbed by a sensitive target. A target molecule would be protected only if an absorbing site were situated directly between the photon and target. An absorbing material external to the cell as a coating would offer protection from irradiation from any direction. Any radiation would have to pass through the coat to reach the target within the cell, and greater thicknesses or more strongly absorbing materials should increase protective effects. Bacteria vary greatly in their production of pigments and other metabolic products which absorb far UV energies, and this may also contribute to the various sensitivities to UV irradiation seen among bacterial species.

Variation in sensitivity to UV killing may have important effects on conclusions based on results of experiments employing certain bacteria as test organisms. For example, E. coli is often used as a standard test organism in air sanitation (Shechmeister, 1968). M. phlei has been reported more resistant than E. coli when both are irradiated on agar surfaces (Collins, 1971) or in 7H9 medium (David, unpublished data) and while in an airborne state (unpublished data from this lab). Also, compared to airborne S. marcescens (Riley and Kaufman, 1971), airborne M. smegmatis, M. phlei are much more resistant to 253.7 nm UV irradiation

(see Table 3). This is probably true of many other species. When a given organism is used to test the effectiveness of air sanitation set-ups incorporating ultraviolet disinfection equipment, care should be exercised when extrapolating to other species of bacteria, for the projected organism may actually behave much differently in the system than the test organism.

Even in "simple" cells such as bacteria, the many interactions between the substances and structures in the cell surely are complex and at this time there is much that is not understood about these processes. It may not be very accurate or even reasonable to attribute a major contribution of any one cause to a response of an organism, even one as simple as a bacterium, to an insult. The sensitivity of any bacteria to radiation is influenced by genetic factors, efficiency of one or more repair mechanisms, and protective substances in or on the cell. During irradiation, the physical environment can affect these factors and also can influence the significance of initial damage to the cell, and, therefore, may affect the response of the cell to an insult.

CHAPTER V

CONCLUSIONS

Based on the results of this investigation, the following conclusions were made:

Certain mycobacteria which have been inactivated by far UV while in an airborne state may show photoreactivation if the cells are immediately exposed to near UV or visible light while on a nutrient agar surface.

Bacteria that have been exposed to 253.7 nm UV while in an airborne state may show species differences in sensitivity to killing.

Relative humidity affects the sensitivity of bacteria to UV, and in general, the lower the humidity, the more sensitive.

Both Mycobacterium phlei and M. smegmatis were much more sensitive to UV irradiation when airborne than when in liquid suspension.

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